Brain and retinal macro- and microvasculature

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CHAPTER 4

Comparative transcriptome analysis of inner blood-retinal barrier and blood-brain barrier in rats

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Abstract

Aims: Although retinal microvessels (RMVs) and brain microvessels (BMVs) share similar blood-neural barriers, studies have reported differential responses to stressors such as diabetes. Researchers have successfully extracted BMVs but not yet RMVs. Therefore, systematic comparison of both microvascular beds including gene expression patterns have not been possible so far.

Methods: In this study, both RMVs and BMVs were mechanically isolated from rats. Total retinal and brain tissues (RT, BT) were collected for comparisons. RNA samples extracted from these four groups were processed on Affymetrix rat 2.0 microarray Chips. The transcriptional profiles of these tissues were then analyzed.

Results: Overall, we found 4,175 differentially expressed genes (DEGs) between RMVs and RT, of which 1,746 genes were significantly higher expressed in RMVs. We also identified 7,245 DEGs between BMVs and BT, of which 3,322 genes were significantly higher expressed in BMVs. Using these DEGs, we comprehensively analyzed the DEGs expression levels and highlighted their involvement in critical functional structures in RMVs and BMVs, such as junctional complex, transporters and signaling pathways.

Conclusion: Our work provides for the first time the transcriptional profiles of rat RMVs and BMVs, which will be helpful to understand why retina and brain microvasculature show different susceptibilities to stressors and might provide new insight for pharmacological interventions.

Introduction

The retina is considered as an extension of the central nervous system. Its inner microvasculature that forms the inner blood-retinal barrier (iBRB), shares characteristics with the brain microvasculature that forms the blood brain barrier (BBB), in terms of anatomy and function (1). Previous studies have demonstrated that in physiological conditions these two types of microvasculature may differ with respect to ATP-binding cassette (ABC) efflux transporters activities, e.g., P-gp (gene symbol ABCB1) and BCRP (gene symbol ABCG2) (2). However, data on possible differences at the level of gene expression are scarce. More interestingly, these two types of microvasculature also display different responses to stressors. For instance, hyperglycemia (high blood glucose) causes pronounced alterations in retinal microvasculature while the brain microvasculature is less susceptible (3, 4). The mechanisms contributing to these differences is yet to be determined.

Retinal vascular endothelial cells (RVECs) and brain vascular endothelial cells (BVECs) have been widely used in vitro as models for gene expression studies in the iBRB and BBB (5-8), in which some intercellular junctions, solute carriers (SLC) and ATP-binding cassette (ABC) transporter genes were found to be expressed (9-13). It should be noted, however, that the pericytes of the microvessels are also required to maintain the structural and functional integrity of the iBRB and BBB. Pericytes surround the abluminal side of microvessel endothelial cells and contribute to the microvessel development, network stabilization and remodeling, and blood flow regulation (14-16). Therefore, to better characterize the blood-neuronal barrier properties, isolated retinal microvessels (RMVs) and brain microvessels (BMVs) consisting of endothelial cells and pericytes are in many respects superior to isolated endothelial cells.

In rats, gene expression profiles of the isolated BMVs have been extensively investigated (17-20). However, the gene expression patterns of the rodent retinal microvasculature are unknown, because of the lack of an effective method for isolating sufficient RMVs. Enzymatic digestion metheods to isolate the RMVs have been reported (3, 21-24), but these vessel preparations were mainly used for analyzing the retinal vasculature morphology or quantifying protein expression levels. In this study, we utilized a mechanical filtration method that was recently developed in our group to isolate both rat RMVs and BMVs while preserving the cellular structure and RNA

integrity (25). To comprehensively elucidate the gene expression profile of microvessels and retinal and brain tissues (RT and BT, respectively), these tissues were collected from the same animals. Transcriptomes of the RMVs, RT, BMVs and BT were then analyzed. Using these transcriptional data, we aimed to identify differentially expressed genes (DEGs) between RMVs / RT and BMVs / BT, and then describe gene expression similarities and differences between RMVs and BMVs, with an emphasis on the junctional complex, membrane transporters and endothelial/pericyte signaling pathways. We hypothesize that these two types of microvessels will display substantial differences in gene expression levels even though they are of the same embryological origin. This heterogeneity at the transcriptome level may translate into differences in physiological responses that may play a role in the differential susceptibility of the iBRB and BBB to stressors like high blood glucose levels.

Materials and Methods

Animals

This study was approved by the Federal Animal Ethics Committee (Karlsruhe, Germany). All experimental procedures complied with the ethical regulations of the Directive 2010/63/EU. Male Wistar rats purchased from Janvier (Isle St- Genest, France) were used in this study. They were housed following a standard 12-hour light/dark cycle in a temperature-controlled environment, and with free access to food and tap water. Six rats (546 ± 17g, body weight) were anesthetized deeply with CO2 inhalation and sacrificed. Brains were dissected by removing meninges, superficial vessels, choroid plexus and white matter. Eyes and brain hemispheres were snap-frozen in liquid nitrogen and stored at -80 °C until use.

Isolation of microvessels

For RMVs isolation, the eyes from individual rats were cryosectioned (HBM500, Microm, Nussloch, Germany). A section of 50 μm was collected for RNA extraction from total retina tissue. The other sections (200 μm, thickness) were transferred into a

glass tube containing 3 ml phosphate-buffered saline (PBS / 1% dextran (Dextran 70,000, Roth); PBS: NaCl 137 mM, KCl 2.7 mM, $Na2HPO_4$ 10 mM, $KH2PO_4$, 1.8 mM, pH 7.4). This retinal preparation was homogenized using a motor-driven homogenizer (Homgen plus, Schuett Biotec, Goettingen, Germany; 60 rpm, 20 upstrokes). The homogenate was then transferred onto an ice-cold density gradient column which contained 3 ml PBS / 31% dextran in the lower phase and 3 ml PBS / 18% dextran in the upper phase. After 15 min centrifugation (1300 g, 4°C), the interphase was carefully collected and diluted into 20 ml PBS solution. Finally, the RMVs were captured after filtering over a 60 µm nylon mesh.

For BMVs isolation, serial sections (thickness, 100 µm) were prepared from an individual hemisphere of which three sections were processed for RNA extraction. The remaining sections were homogenized in the same way as for RMVs isolation. Subsequently, the suspension was mixed with PBS / 1% dextran until 50 ml and centrifugated for 10 min (438 g, 4°C). The pellet was resuspended into 22 ml PBS / 18% dextran and centrifuged again for 15 min (4400 g, 4°C). The pellet was resuspended into 7 ml PBS / 1% dextran. The subsequent step for BMVs isolation were identical as described for the RMVs isolation.

RNA preparations and quality control

The samples were immersed in 350µl lysis buffer (RLT solution; Qiagen, Germany) plus 3.5µl mercaptoethanol (Sigma), and then pulled ten times through a 22-gauge needle. Total RNA was obtained from individual microvessel samples or control retinal and brain tissues with RNeasy® Plus Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration and quantity were determined using the Agilent 6000 Pico kit (RMVs and BMVs), or Agilent Nano kit (brain and retinal tissues) on an Agilent 2100 bioanalyzer (Agilent Technologies, USA). All RNA samples (integrity number > 7.0) were used for microarray processing (Table S1).

Microarray processing

For each sample, 1 ng of total RNA was amplified using the GeneChip® WT Pico Reagent Kit (Affymetrix) according to the manufacturer's protocol. 20 µg of cRNA was

used as input for the second cycle of cDNA reaction. 5.5µg of single-stranded cDNA was used as input for the fragmentation reaction. The Affymetrix Genechip WT Terminal labeling kit was used for fragmentation and biotin labeling. Finally, the samples were hybridized to the whole-transcriptome Rat Gene 2.0 ST microarrays (Affymetrix) on the Genechip Fluidics Station 450 (Affymetrix), scanned using the Genechip Scanner 7G (Affymetrix) and the raw intensity values stored in CEL files by the GeneChip® Operating Software (Affymetrix). These raw CEL files were normalized using the Affymetrix® Expression Console Software (version 4.0, Affymetrix) and the adjusted intensity values were transformed to log2 format. The complete microarray dataset is available at Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE110675.

Microarray data analysis

The microarray data were analyzed with R software using LIMMA package (version 3.02; R Development Core Team, 2013). The obtained LIMMA False Discovery Rate (FDR) adjusted p-value calculated by the Benjamini-Hochberg approach, and fold change (FC) were used as a cut-off to identify the DEGs. To assess the gene expression profiles of RMVs, we firstly identified DEGs between RMVs and RT. Genes with an adjusted $p < 0.05$ and $FC_{(RMVs\text{-}RT)} > \pm 1.5$ were considered statistically significant. In a second pairwise comparison, we identified DEGs between RMVs and BMVs. Genes with an adjusted $p < 0.05$ and $FC_{(RMVs\text{-}BMVs)} > \pm 1.5$ were selected. Also, a comparison between RT and BT was additionally performed. Finally, genes with an adjusted $p < 0.05$ and $\Delta FC = FC_{(RMVs\text{-}BMVs)} - FC_{(RT\text{-}BT)} > \pm 1.5$ were considered statistically significant. To investigate the functional classifications of these DEGs, biological process categories were analyzed using Gene Ontology (GO) Consortium (http://geneontology.org/). Pathways analysis was performed using the PANTHER Classification System (26).

Results

A total of 20,743 genes were measured using Affymetrix rat 2.0 microarrays for the transcriptome analysis of microvessels (RMVs and BMVs) and tissue samples (RT and BT). These genes were analyzed by principal components analysis (PCA). Using the principal component 1 and 2 (accounting for 95% and 3% of the variations, respectively), we identified four distinct clusters in which brain and retinal tissues were clearly separated from microvessels while the RMVs and BMVs remained close to each other (Fig. 1).

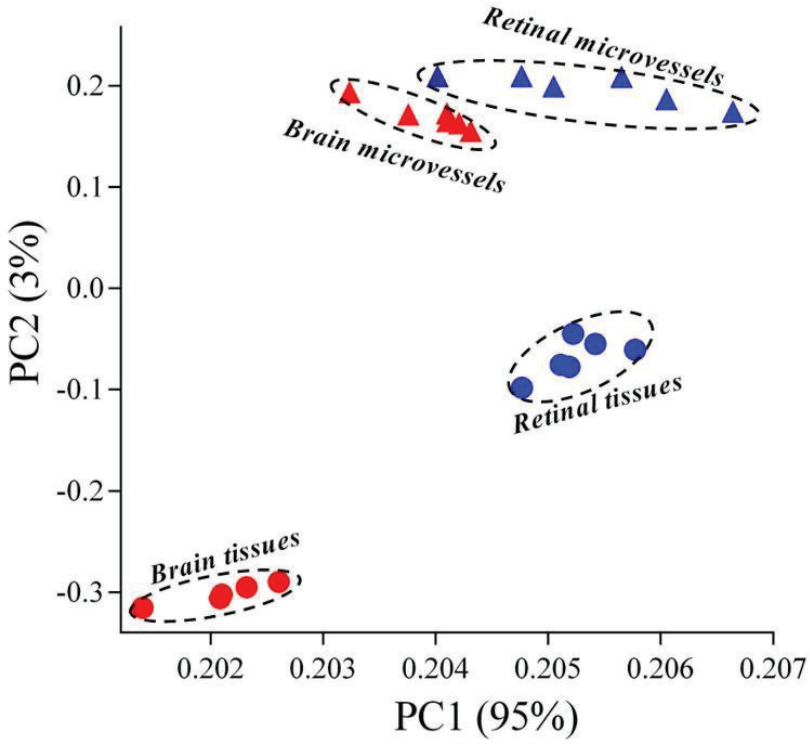
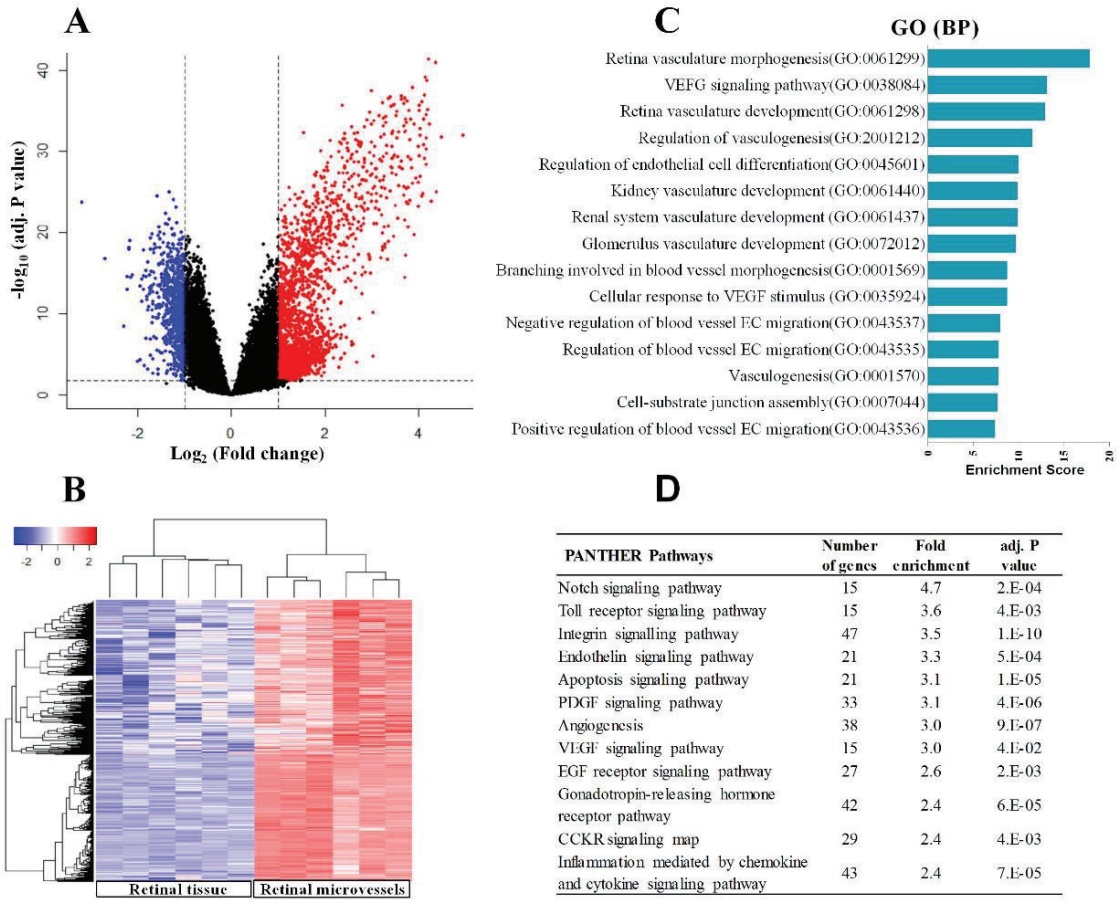


Figure 1: Principal component analysis (PCA) performed on whole transcriptome genes identified from RMVs, BMVs, RT and BT of rats. The first component (PC1) with a variance of 95% is on the X-axis and the second component (PC2) with a variance of 3% is on the Y-axis. Each dot represents one tissue sample. Red color represents brain samples; Blue color represents retinal samples; Triangle represents microvessel samples; Circle represents microvessel samples;

Overall transcriptional comparison between RMVs and RT

In the transcriptional comparison between RMVs and RT we identified 4,175 significantly DEGs (FC> 1.5 and FDR adjusted p< 0.05), of which 1,746 genes were

higher expressed in RMVs, while 2,429 genes were lower expressed in RMVs than in RT (Fig. 2A). Hierarchical cluster analysis of these significantly higher expressed genes showed clear separation between the groups (Fig. 2B). To investigate the functional classifications of those RMVs enriched genes, gene ontology (GO) biological process categories were analyzed. The top 15 GO biological processes are displayed in figure 2C (ranked by enrichment score) and represent mainly vasculature-related biological processes including "retina vasculature morphogenesis" and "retina vasculature development". To investigate enriched pathways the PANTHER database was utilized. This analysis identified 12 enriched pathways in RMVs, e.g., "Notch signaling pathway", "Toll receptor signaling pathway" and "PDGF signaling pathways" (Fig 2D).



PANTHER Pathways	Number of genes	Fold enrichment	adj. P value
Notch signaling pathway	15	4.7	2.E-04
Toll receptor signaling pathway	15	3.6	4.E-03
Integrin signalling pathway	47	3.5	1.E-10
Endothelin signaling pathway	21	3.3	5.E-04
Apoptosis signaling pathway	21	3.1	1.E-05
PDGF signaling pathway	33	3.1	4.E-06
Angiogenesis	38	3.0	9.E-07
VEGF signaling pathway	15	3.0	4.E-02
EGF receptor signaling pathway	27	2.6	2.E-03
Gonadotropin-releasing hormone receptor pathway	42	2.4	6.E-05
CCKR signaling map	29	2.4	4.E-03
Inflammation mediated by chemokine and cytokine signaling pathway	43	2.4	7.E-05

Figure 2: Gene expression comparison between RMVs and retinal tissues in rats (n=6). **(A)** Volcano plot for the RMVs versus RT whole transcriptomes. The red dots indicate the genes that are significantly higher expressed (FC> 1.5 and FDR adjusted p< 0.05) in RMVs compared to RT, while blue dots indicate the lower expressed (FC< -1.5 and FDR adjusted p< 0.05) in RMVs compared to RT. **(B)** Hierarchical cluster analysis for genes that are significantly higher expressed (FC> 1.5 and FDR adjusted p< 0.05) in RMVs than in RT. **(C, D)** Top 15

gene ontology (GO) biological processes (BP) and 12 PANTHER pathways identified from the RMVs enriched genes. All the biological processes shown are ranked by enrichment score with a Bonferroni adjusted $p< 0.05$.

Overall transcriptional comparison between the RMVs and BMVs

We identified a total of 7,245 DEGs (FC> ±1.5 and FDR adjusted $p< 0.05$) between BMVs and BT, of which 3,321 genes were significantly higher expressed in BMVs compared to BT (Fig. S1). Based on the overlap between the BMVs and RMVs enriched genes (3,321 and 1,746, respectively), we identified 1,516 common genes (accounting for 87% RMVs genes) (Fig. 3A). Among these common genes, we identified 12 PANTHER pathways, e.g., "Notch signaling pathway", "VEGF signaling pathway" and "PDGF signaling pathways" (Tab. S2). Moreover, we identified 1,805 genes that were enriched in BMVs, and 230 genes that were enriched in RMVs (Fig. 3A). To investigate the functional classifications of these three groups, GO biological process categories were analyzed. The top 15 GO biological processes of the three groups are displayed in figures 3B, C and D (ranked by enrichment score), e.g., 'VEGF pathway" and "retinal vasculature development" for the common genes.

Gene expression pattern of junctional complex in RMVs and BMVs

To further characterize the gene expression profiles of RMVs and BMVs, we highlighted the gene groups related to the junctional complex, membrane transporters and endothelial / pericyte signaling pathways. For the junctional complex, we found that occludin (Ocln), junctional adhesion molecule B (Jam2) and C (Jam3), claudin-5 (Cldn-5) and VE-cadherin (Cdh5) mRNA were high and equally expressed in both microvessel beds (Tab. 1). However, the expression level of tight junction protein-1 (ZO-1), Gap junction alpha-1 protein (Cx43), Gap junction alpha-4 protein (Cx37), Gap junction alpha-5 protein (Cx40) and Catenin alpha-1/α-catenin (Ctnna1) were significantly lower in RMVs compared to BMVs (Tab. 1).

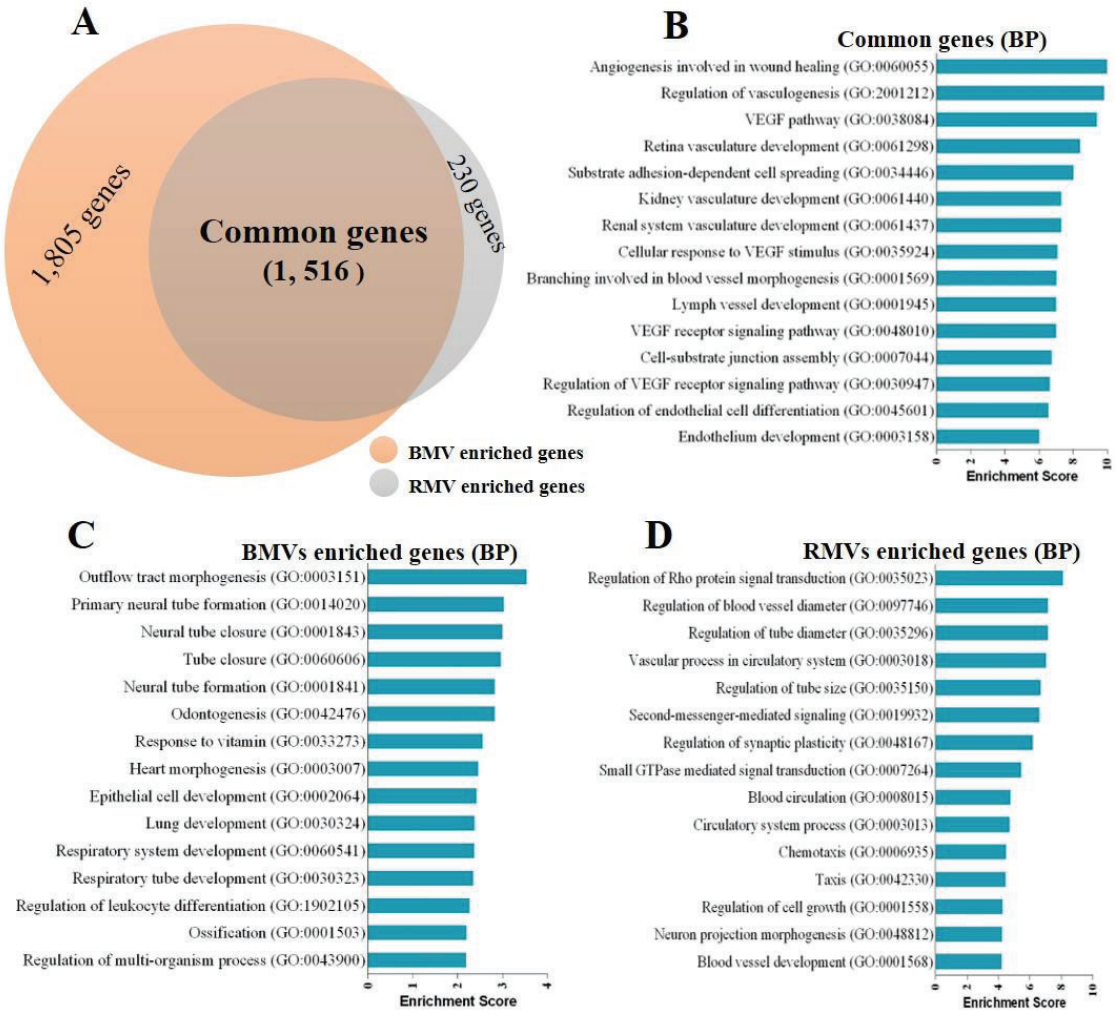


Figure 3: Gene expression comparison between RMVs and BMVs in rats. **(A)** Venn diagram showing the number of RMVs and BMVs genes that are significantly higher expressed compared to retina tissue (1746 genes) and brain tissue (3321 genes), respectively. Overlap of these genes is indicated by: common genes. Top 15 gene ontology (GO) biology processes (BP) identified from the 1516 common genes **(B)**, the 1,805 BMVs enriched genes **(C)** and 230 RMVs enriched genes **(D).** All the biological processes shown are ranked by enrichment score with a Bonferroni adjusted $p< 0.05$.

Gene expression pattern of membrane transporters in RMVs and BMVs

We identified 355 solute carrier (SLC) and 47 ATP-binding cassette (ABC) transporter genes from the isolated RMVs, of which 56 genes were significantly higher expressed in RMVs compared to RT and 96 genes were significantly higher expressed in BMVs compared to BT. In comparison with BMVs, Slc2a4 (Glut-4), Slc7a2 (CAT2), Slc16a2 (MCT8), Slc22a6 (OAT1), Slc22a8 (OAT3), Slc26a10, Slc28a3, Slc52a3 mRNA were significantly lower expressed in RMVs, whereas Slc16a1 (MCT1) was significantly higher expressed (Tab. 2). For the ABC transporters, we found that Abcb1a (P-gp), Abcg2 (BCRP), Abcc1 (MRP1), Abcc4 (MRP4), Abcd1 (ALD) and Abcc9 mRNA were highly and equally expressed in the two types of microvessel beds, while the expression level of Abca9 was significantly lower in RMVs compared to in BMVs (Tab. 3).

Gene expression pattern of signaling pathways in RMVs and BMVs

For the signaling between endothelial cells and pericytes, we found that genes of the PDGF-B/PDGFRβ pathway, Tie2/Angiopoietins pathway, TGF-β signaling pathway and Notch signaling pathway were highly enriched in the two types of microvessel beds (Tab. 4). In comparison with BMVs, the expression level of angiopoietin 1 (Ang-1), Jagged-2 (Jag-2), TGFβ mRNA was significantly lower in RMVs (Tab. 4).

Table 1. Expression of intercellular junctions in rat RMVs and BMVs, RT and BT.

Gene Symbol	Full gene name	RT	RMVs	BT	BMVs	*RMVs. vs. RT*		*RMVs. vs. BMVs*		*RT. vs. BT*	
						log_2FC	*adj. P*	*log_2FC*	*adj. P*	*log_2FC*	*adj. P*
Ocln*	Occludin	6.4±0.13	9.7±0.17	7.4±0.06	10.8±0.04	3.3	2.E-27	-1.1	8.E-10	-1.0	3.E-08
Cldn5*	Claudin-5	9.4±0.13	13.1±0.1	10.3±0.04	13.4±0.01	3.6	7.E-34	-0.3	2.E-02	-0.9	6.E-10
Jam2*	Junctional adhesion molecule B	10.9±0.02	12±0.05	10.2±0.02	12.2±0.04	1.1	5.E-20	-0.2	8.E-03	0.7	3.E-12
Jam3*	Junctional adhesion molecule C	6.8±0.07	7.5±0.08	7.7±0.03	8.2±0.09	0.7	2.E-07	-0.7	2.E-07	-0.8	8.E-11
Cdh5*	VE-cadherin	7.1±0.06	11.4±0.1	8.1±0.11	12±0.01	4.4	2.E-37	-0.6	1.E-05	-1.0	3.E-11
ZO-1*#	Tight junction protein-1	10.8±0.03	11.6±0.09	10.6±0.03	12.2±0.03	0.8	3.E-15	-0.7	4.E-12	0.1	9.E-02
Ctnna1#	Catenin alpha-1/α-catenin	8.1±0.07	8.6±0.12	8.1±0.03	9.3±0.01	0.5	1.E-05	-0.7	9.E-08	0.0	9.E-01
Cx37*#	Gap junction alpha-1 protein	7.4±0.36	9.0±0.10	11.5±0.02	10.9±0.06	1.6	2.E-08	-1.9	2.E-09	-4.1	3.E-21
Cx43*#	Gap junction alpha-4 protein	6.9±0.07	10.1±0.07	6.9±0.10	10.8±0.08	3.2	6.E-27	-0.7	5.E-05	0.0	1.E+00
Cx40#	Gap junction alpha-5 protein	6.2±0.09	6.9±0.05	6.1±0.13	8.6±0.09	0.7	5.E-06	-1.7	7.E-18	0.1	6.E-01

Results are mean ± SEM (log2 transformed microarray intensity, n=6). RT, retinal tissue; RMVs, retinal microvessels; BT, brain tissue; BMVs, brain microvessels; adj. P, false discovery rate adjusted p value; log_2FC, log2 transformed fold change. *significant compared to RT; #significant compared to BMVs.

Table 2. Expression of solute carrier transporters in rat RMVs and BMVs, RT and BT.

Gene symbol	RT	RMVs	BT	BMVs	*RMVs. vs. RT*		*RMVs. vs. BMVs*		*RT. vs. BT*	
					log_2FC	*adj. P*	*log_2FC*	*adj. P*	*log_2FC*	*adj. P*
Slc2a1*	11.8±0.03	13.4±0.05	11.1±0.03	13.5±13.5	1.61	3.E-22	-0.14	2.E-01	0.7	8.E-10
Slc2a4*#	6.3±0.07	7.4±0.08	6.2±0.13	8.4±8.4	1.08	1.E-10	-1.01	7.E-10	0.1	6.E-01
Slc7a1*	8.6±0.2	11.9±0.12	9.5±0.04	12.5±12.5	3.32	9.E-23	-0.57	1.E-02	-0.9	3.E-05
Slc7a2*#	8.2±0.16	9±0.07	8.8±0.06	10.8±10.8	0.80	3.E-06	-1.79	2.E-16	-0.6	1.E-04
Slc7a5*	11±0.07	11.8±0.06	9.7±0.08	12.3±12.3	0.78	7.E-07	-0.44	4.E-03	1.3	3.E-13
Slc43a1*	5.1±0.11	6.9±0.14	5.5±0.17	7.5±7.5	1.83	2.E-13	-0.66	2.E-03	-0.5	2.E-02
Slc7a15*	6.3±0.14	8.2±0.13	6.3±0.07	8.6±8.6	1.84	2.E-16	-0.41	2.E-02	0.1	7.E-01
Slc38a5*#	6.1±0.1	9.6±0.19	6.3±0.16	10.4±10.4	3.47	3.E-18	-0.82	5.E-03	-0.2	6.E-01
Slc16a1*	10.4±0.05	11.4±0.11	8.7±0.09	9.7±9.7	1.05	1.E-04	1.75	4.E-09	1.7	7.E-09
Slc16a2*#	7.3±0.09	8.6±0.13	8.5±0.1	10.9±10.9	1.28	6.E-13	-2.28	9.E-23	-1.2	2.E-12
Slc16a4*	4.8±0.11	7.4±0.15	5.1±0.07	8.4±8.4	2.62	3.E-20	-0.99	1.E-06	-0.3	1.E-01
Slc26a10#	5.9±0.07	6.4±0.07	6.1±0.06	9.2±9.2	0.52	2.E-04	-2.82	1.E-28	-0.2	2.E-01
Slc22a5*	7±0.14	8.7±0.11	7.1±0.11	9.1±9.1	1.75	1.E-11	-0.33	2.E-01	-0.1	6.E-01
Slc22a6#	6.2±0.15	6.3±0.08	6.7±0.05	10.2±10.2	0.12	7.E-01	-3.90	7.E-25	-0.5	3.E-02
Slc22a8*#	7±0.28	9.7±0.14	7.8±0.03	11.5±11.5	2.67	2.E-12	-1.82	1.E-07	-0.8	9.E-03
Slc28a3*#	5.9±0.08	6.8±0.11	5.8±0.05	7.5±7.5	0.91	1.E-07	-0.71	2.E-05	0.0	8.E-01
Slc52a3*#	6.3±0.05	9.4±0.14	6.8±0.05	10.7±10.7	3.08	4.E-26	-1.35	6.E-12	-0.5	4.E-03
Slco1a2*	7.5±0.13	11.4±0.11	8.6±0.08	12.1±12.1	3.87	2.E-24	-0.75	1.E-03	-1.1	3.E-06
Slco1c1*	9.5±0.06	12.8±0.11	10.7±0.04	13.3±13.3	3.29	2.E-17	-0.54	8.E-02	-1.3	1.E-05
Slco2b1*	7.1±0.08	9.2±0.1	8.1±0.07	10.5±10.5	2.19	4.E-22	-1.24	1.E-12	-1.1	1.E-10

Results are mean ± SEM (log2 transformed microarray intensity, n=6). RT, retinal tissue; RMVs, retinal microvessels; BT, brain tissue; BMVs, brain microvessels; adj. P, false discovery rate adjusted p value; log_2FC, log2 transformed fold change. *significant compared to RT; #significant compared to BMVs.

Table 3. Expression of ABC transporters in rat RMVs and BMVs, RT and BT.

Gene	RT	RMVs	BT	BMVs	*RMVs. vs. RT*		*RMVs. vs. BMVs*		*RT. vs. BT*	
					log_2FC	*adj. P*	*log_2FC*	*adj. P*	*log_2FC*	*adj. P*
Abcb1*	7±0.14	10.7±0.14	8±0.07	11.6±11.6	3.69	5.E-23	-0.96	6.E-05	-1.0	1.E-05
Abcc1*	8.8±0.04	9.5±0.09	7.3±0.08	8.7±8.7	0.63	6.E-05	0.73	6.E-06	1.5	4.E-15
Abcc4*	6.7±0.08	9.2±0.14	7.1±0.08	10.7±10.7	2.49	9.E-22	-1.51	3.E-13	-0.4	2.E-02
Abcc6*	6±0.07	6.6±0.03	5.7±0.07	7.1±7.1	0.57	3.E-05	-0.50	3.E-04	0.3	2.E-02
Abcc9*	7.4±0.13	11.4±0.15	7.5±0.09	11±11	4.03	7.E-30	0.45	1.E-02	-0.1	6.E-01
Abcg2*	5.4±0.16	9.3±0.14	6.6±0.22	10.5±10.5	3.90	2.E-18	-1.15	4.E-04	-1.2	2.E-04
Abcd1*	7.3±0.08	8.9±0.1	8.1±0.12	9.6±9.6	1.57	1.E-16	-0.71	2.E-06	-0.8	2.E-07
Abca9#	6.3±0.09	6.2±0.1#	5.8±0.16	8.8±8.8	-0.16	4.E-01	-2.64	7.E-23	0.5	2.E-03

Results are mean ± SEM (log2 transformed microarray intensity, n=6). RT, retinal tissue; RMVs, retinal microvessels; BT, brain tissue; BMVs, brain microvessels; adj. P, false discovery rate adjusted p value; log_2FC, log2 transformed fold change. *significant compared to BMVs; #significant compared to BMVs.

Table 4. Expression of signaling genes between endothelial cells and pericytes in rat RMVs and BMVs, RT and BT.

Gene	RT	RMVs	BT	BMVs	*RMVs. vs. RT*		*RMVs. vs. BMVs*		*RT. vs. BT*	
					log_2FC	*adj. P*	*log_2FC*	*adj. P*	*log_2FC*	*adj. P*
Pdgfβ*	6.5±0.05	9.3±0.14	7.2±0.07	10±0.06	2.8	7.E-23	-0.7	4.E-05	-0.7	9.E-05
Pdgfbrβ*	8±0.08	11.4±0.09	8.3±0.06	11.6±0.06	3.4	4.E-33	-0.2	9.E-02	-0.4	6.E-03
Tie2*	7.5±0.11	11.2±0.11	8.6±0.06	12.1±0.03	3.7	1.E-33	-0.9	1.E-09	-1.1	4.E-12
Ang-1#	5.5±0.23	5.9±0.12	6.1±0.1	7.3±0.08	0.4	6.E-02	-1.4	2.E-08	-0.6	4.E-03
Ang-2*	6.9±0.1	8±0.13	5.9±0.16	8.6±0.06	1.1	4.E-07	-0.6	2.E-03	1.1	2.E-07
TGFβ*#	6.5±0.06	8.6±0.07	6.9±0.13	9.9±0.04	2.1	3.E-23	-1.3	1.E-14	-0.5	5.E-04
Tgfbr2*	6.8±0.07	9.5±0.13	7.3±0.08	10.3±0.05	2.7	6.E-27	-0.8	2.E-07	-0.5	6.E-04
Tgfbr3*	7.5±0.09	10±0.13	8.6±0.04	11.5±0.04	2.5	6.E-27	-1.5	1.E-17	-1.1	5.E-13
Alk-1*	6.4±0.08	10.1±0.08	6.9±0.07	10.9±0.03	3.7	1.E-33	-0.8	2.E-07	-0.5	2.E-04
Alk-5	7.7±0.1	7.5±0.06	7.7±0.1	8±0.06	-0.2	3.E-01	-0.5	1.E-03	0.0	1.E+00
Dll4*	6.4±0.14	9.5±0.09	8.6±0.06	10.2±0.16	3.1	8.E-20	-0.7	3.E-09	-2.2	6.E-02
Jag-1*	9.8±0.05	10.7±0.04	8.2±0.05	10.9±0.04	0.9	3.E-11	-0.2	8.E-13	1.6	1.E-27
Notch1*	8.4±0.08	10.5±0.1	6.9±0.05	11.4±0.05	2.1	1.E-22	-0.9	1.E-10	-0.5	6.E-03
Notch3*	7.4±0.07	10.8±0.18	7.6±0.12	11.5±0.06	3.4	2.E-27	-0.7	2.E-02	-0.2	2.E-01

Results are mean ± SEM (log2 transformed microarray intensity, n=6). RT, retinal tissue; RMVs, retinal microvessels; BT, brain tissue; BMVs, brain microvessels; adj. P, false discovery rate adjusted p value; log_2FC, log2 transformed fold change. *significant compared to RT; #significant compared to BMVs.

Discussion

In this study, we comprehensively analyzed the whole transcriptional profile of isolated RMVs and BMVs, RT and BT from the same rats. To our knowledge, this is the first study in which a detailed expression analysis was performed in isolated rat RMVs. Using the transcriptional comparison to RT, we identified 1,746 RMVs enriched genes which were mainly associated to vascular biological processes. This is also the first report in which the whole transcriptomes of two embryologically intimately related microvessel beds, RMVs and BMVs, were characterized and compared in parallel. Although, as expected, their overall gene expression profiles displayed two close, but distinct clusters in the PCA, these two types of microvessels also showed considerable differences at the gene expression level of the junctional complex, membrane transporters and endothelial/pericyte signaling pathways.

The permeability of both iBRB and BBB is mainly governed by the interendothelial junctional complexes that consists of tight junctions, adherens junctions and gap junctions. In our study, both RMVs and BMVs expressed high levels of occludin, claudin-5, Jam2, Jam3, ZO-1, Cx43, Cx40, Cx37 and VE-cadherin, suggesting that tight intercellular junctions are important features in these isolated microvessels. For BMVs, this is consistent with a previous report, in which junctional gene expression was observed (17). Expression of occludin and claudin-5 was also observed in RVEC cultures (9, 11). The tightness of the iBRB and BBB is directly related to the expression level of junctional genes, which also determines the susceptibility to endogenous or exogenous insults. For example, Tien et al found that the downregulation of Cx43 in the retina by Cx43 siRNA or streptozotocin (STZ) injection, contributed to compromised retinal vascular homeostasis (27). More recently, using immunohistochemistry and vasomotor response assessment, Ivanova, et al demonstrated that Cx43 gene expression level in rat retinal capillaries was significantly decreased by hyperglycemia and that this decrease contributed to the vasomotor decline of the inner retinal capillaries(28). Furthermore, it has been demonstrated that *in vitro* downregulation of the Cx43 / ZO-1 complex in RVECs or BVECs contributes to the breakdown of the iBRB and BBB (29, 30). In this study, we demonstrate that in physiological conditions the expression levels of Cx43 and ZO-1 were significantly lower in RMVs compared to BMVs. Based on these observations, we speculate that the

lower baseline expression of Cx43 and ZO-1 expression in RMVs might contribute to a higher susceptibility to e.g. hyperglycemia-induced damage of the iBRB compared to the BBB.

The transcellular route in the iBRB is mainly controlled by membrane transporters. Systematic studies of the transporter gene expression patterns in the rodent iBRB have been limited until recently due to the difficulty in isolating microvessels of sufficient quality and purity to extract RNA. Instead of RMVs, retinal vascular endothelial cells (RVECs) and a conditionally immortalized retinal capillary endothelial cell line (TR-iBRB2) have been used as iBRB models. Using these model, Tachikaya et al identified 14 efflux transporter mRNAs that were expressed in RVECs of which 5 (Abca9, Abcb1, Abcc4, Abcc6 and Abcg2) were highly expressed in RVECs (11). Other studies have shown that Slc7a1 (CAT1), Slc7a5 (LAT-1), Slc16a1 (MCT-1) and Slc22a5 (OCTA2) mRNA were expressed in the TR-iBRB2 cell line (31-34). In our study employing whole transcriptome analysis, we identified 355 SLC and 47 ABC transporter genes in the isolated RMVs of which 56 genes were significantly higher expressed in RMVs compared to RT and 96 genes were significantly higher expressed in BMVs compared to BT. Our findings expand the database of transporter genes expressed in the iBRB and also increases our knowledge of similarities and differences between iBRB and BBB. For instance, we found that the expression level of Glut-1, which is a major transporter of glucose across the barriers, was expressed at comparable levels in both RMVs and BMVs. In contrast, in a previous study it was described that Glut-1 gene expression was higher in rat RMVs than BMVs and that hyperglycemia downregulated its expression only in RMVs (3). In this previous study, RMVs were isolated by incubation with distilled water (1 hour), followed by DNase I digestion while the BMVs were isolated by a mechanical procedure. These different isolation procedures may affect the analysis outcome. In our study, both RMVs and BMVs were isolated by virtually the same mechanical method allowing direct comparison of the microvessels. Our data also show that the insulin-sensitive glucose transporter (Glut-4), which is also crucial in regulating glucose transport into neuronal tissues (35), was significantly lower expressed in RMVs compared to BMVs. Studies by Ngarmukos et al demonstrated that Glut-4 was expressed in the BBB and it was suggested that Glut-4 participates in brain sensing of blood glucose concentrations (36). We therefore speculate that RMVs may be less sensitive compared to BMVs to changes in blood

glucose concentrations, which subsequently may lead to impaired glucose control in the retina.

In recent years, it has been demonstrated that there are several critical signaling pathways between endothelial cells and pericytes such as PDGFβ / PDGFRβ signaling, Tie2 / Angiopoietins signaling, TGF-β signaling and Notch signaling (15, 37). The communication between endothelial cells and pericytes in BMVs plays a key role in the formation and maintenance of the BBB integrity (38). In this study, the majority of genes of these signaling pathways were also detected in RMVs. PANTHER pathway analysis revealed that Notch signaling and PDGF signaling pathways were significantly overrepresented in the common genes of RMVs and BMVs. We also quantified the expression level of these genes and demonstrated that both Ang1 and TGFβ were less expressed in RMVs than in BMVs. Previous studies have demonstrated that activation of the Tie2 / Ang-1 pathway promotes endothelial cell stabilization (39). The biological effects of TGFβ depends on its receptors since activation of the TGFβ / Alk-5 pathway promotes vessel maturation whereas the TGFβ / Alk-1 pathway has an opposite effect (15). In our study, none of these signaling pathways were found to be entirely and quantitatively different between RMVs and BMVs, suggesting that the endothelial-pericyte signaling communication in RMVs and BMVs is comparable.

In summary, we provided for the first time the transcriptional profiles of rat retinal microvasculature and compared with brain microvasculature from the same rats. Our study increases the knowledge of gene expression patterns in retina and brain vascular systems which is instrumental to understand why retina and brain show different susceptibilities to stressors and might provide new targets for specific pharmacological interventions.

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Author contributions

Schilling L., Li Y. and Moshage H. conceived the study and designed the experiments. Li Y., Schilling L., De La Torre C performed the experiments. Li Y., Kamps JA., Faiz A. and Wang J. analyzed the data, discussed the results and wrote the manuscript. All authors commented on the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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Supplementary results

Table S1: Total RNA content and integrity assessment

Total RNA	RMVs	BMVs	RT	BT
Conc.	1.4±0.3	6.2±1.5	19.1±1.6	77.5±5.1
RIN	9.4±0.2	9.5±0.1	8.5±0.3	8.8±0.2

Results are mean ± SEM (n= 6). Conc., concentration of total RNA given in ng/µl; RIN, RNA integrity number; RT, retinal tissue; RMVs, retinal microvessels; BT, brain tissue; BMVs, brain microvessels.

Table S2: Pathway analysis for the common genes between RMVs and BMVs.

PANTHER Pathways	Number of genes	Fold enrichment	P value
Notch signaling pathway	14	4.9	3.E-04
Toll receptor signaling pathway	14	3.7	6.E-03
Integrin signaling pathway	46	3.7	2.E-11
Apoptosis signaling pathway	28	3.2	2.E-05
Angiogenesis	35	3.1	2.E-06
VEGF signaling pathway	14	3.1	5.E-02
Endothelin signaling pathway	16	2.8	5.E-02
PDGF signaling pathway	27	2.8	5.E-04
Alzheimer disease-presenilin pathway	21	2.6	2.E-02
EGF receptor signaling pathway	24	2.6	7.E-03
Inflammation mediated by chemokine and cytokine signaling pathway	39	2.4	2.E-04
Gonadotropin-releasing hormone receptor pathway	37	2.3	5.E-04

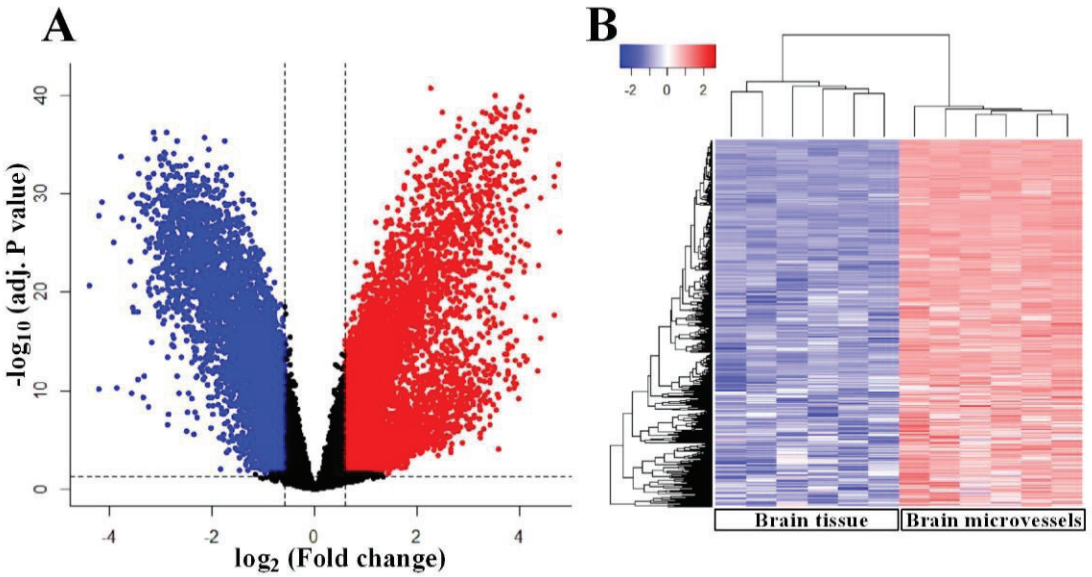

Figure S1: Gene expression comparison between BMVs and brain tissue in rats (n= 6). **(A)** Volcano plot for the BMVs versus BT whole transcriptomes. The red dots indicate the genes that are significantly higher expressed (FC> 1.5 and adjusted p< 0.05) in BMVs compared to BT, while blue dots indicate the lower expressed (FC< -1.5 and adjusted p< 0.05) in BMVs compared to BT. **(B)** Hierarchical cluster analysis for genes that are significantly higher expressed (FC> 1.5 and adjusted p< 0.05) in BMVs than in BT.